

## CLAIMS

1. A  $\beta$  sheet multimeric cytokine whose sequence has been altered by mutating a residue in a monomer component of the multimeric cytokine protein so as to improve the free energy of the monomer or of the multimeric complex relative to the wild-type unmutated monomer component so as to be more stable than the wild-type, unaltered cytokine protein, wherein said mutated residue is non-conserved between homologous members of the cytokine family.
2. A cytokine according to claim 1, which is a member of the TNF ligand family.
3. A cytokine according to claim 2, which is TRAIL.
4. A cytokine according to claim 3, which is mutated in the soluble C-terminal portion of the molecule.
5. A cytokine according to any preceding claim, which is mutated at one or more of the following positions:
  - a) a non-conserved residue at the surface of the monomer component of the multimeric cytokine;
  - b) a non-conserved residue close to the interface between two of the monomer components of the multimeric cytokine;
  - c) for trimeric cytokines, a non-conserved residue along the central trimeric axis;
  - d) a miscellaneous residue whose mutation is energetically favourable.
6. A cytokine according to claim 5, which is mutated in the external loop that connects that C and D anti-parallel beta strands (the CD loop), following the notation according to Eck (Eck et al., J. Biol. Chem. 267, 2119-2122 (1992)).
7. A cytokine according to claim 5 part a), which is mutated at one or both positions 194 and 196.
8. A cytokine according to claim 7, which is a TRAIL mutant containing the mutations E194I and/or I196S.
9. A cytokine according to claim 5 part b), which is mutated at one or more of the positions 125, 163, 185, 187, 232, 234, 237, 203, 205, 239, 241, 271, 274.
10. A cytokine according to claim 9, which is a TRAIL mutant containing one or more of the mutations D203I, Q205M and Y237F.

11. A cytokine according to claim 5 part c), which is mutated at one or more of positions 227, 230 and 240.
12. A cytokine according to claim 11, which is a TRAIL mutant containing the mutation R227M.
- 5 13. A cytokine according to claim 11, which is a TRAIL mutant containing the mutation C230S and Y240F.
14. A cytokine according to claim 5 part d), which is mutated at one or more of the positions 123, 272, 225, 280, 163, 123 and 208.
- 10 15. A cytokine according to claim 14, which is a TRAIL mutant containing the mutation S225A.
16. A cytokine which is mutated at more than one position as listed in claim 5, parts a) to d).
17. A cytokine according to claim 16, which is a TRAIL mutant containing the mutations E194I, I196S and S225A.
- 15 18. A cytokine according to any one of claims 1-17, wherein the described mutations are introduced into a soluble form of the cytokine.
19. A cytokine according to claim 18, which is a TRAIL mutant comprising residues 114-281.
- 20 20. A  $\beta$  sheet multimeric cytokine with selectivity for a target receptor, in which one or more amino acids in the cytokine that are located in the receptor-binding interface are substituted for replacement residues that include amino acid side-chain conformations that are predicted to fit into the binding interface with the target receptor so as to provide an increase in binding affinity and selectivity/specificity of the cytokine protein for that target receptor, provided that these are not residues interacting with amino acids that are conserved among receptors bound by the cytokine protein.
- 25 21. A  $\beta$  sheet multimeric cytokine according to claim 20 which has altered affinity for a particular target receptor.
22. A  $\beta$  sheet multimeric cytokine with selectivity for two or more target receptors wherein selectivity for a first target receptor is achieved by substituting one or more amino acids in the cytokine for replacement residues so as to decrease affinity for one or more different target receptors, provided that these are not residues interacting with amino acids that are conserved among receptors bound by the cytokine protein.
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23. A cytokine according to claim 21 or claim 22, which is mutated at one or more of the positions 131, 269, 130, 160, 218, 220, 149, 155, 214, 195, 191 and 267 in the cytokine.
24. A cytokine according to any one of claims 20 to 23, which is a member of the TNF ligand family.
- 5 25. A cytokine according to claim 24, which is TRAIL.
26. A cytokine according to claim 25, which has superior selectivity for the DR5 (TRAIL-R2) or DR4 (TRAIL-R1) over the decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4).
- 10 27. A cytokine according to claim 25, which has superior selectivity for the death receptor 5 (TRAIL-R2) over selectivity for the death receptor 4 (TRAIL-R1).
28. A cytokine according to claim 27, which contains one or more of the mutations G131R, D269H, D269K, D269R, R130E, G160K, D218R, G160M, I220M, I220H, R149D, R149H, E155M, T214R, E195R, R191E and D267R.
29. A cytokine according to claim 27, which contains the mutations G160M or D269H.
- 15 30. A cytokine according to claim 27, which contains the mutations D269H and T214R.
31. A cytokine according to claim 27, which contains the mutations D269H and E195R.
32. A cytokine according to claim 27, which contains the mutations R191E and D267R.
33. A cytokine according to claim 25, which has superior selectivity for the death receptor 4 (TRAIL-R1) over selectivity for the death receptor 5 (TRAIL-R2).
- 20 34. A cytokine according to claim 33, which contains one or more of the mutations D218Y, D218E, D218K, D218H and D218F.
35. A  $\beta$  sheet multimeric cytokine with selectivity for a target receptor whose sequence has been altered by;
  - 25 a) mutating a residue in a monomer component of the multimeric cytokine protein so as to improve the free energy of the monomer or of the multimeric complex relative to the wild-type unmutated monomer component, wherein said mutated residue is non-conserved between homologous members of the cytokine family, so as to be more stable than the wild-type, unaltered cytokine protein, and
  - 30 b) substituting one or more amino acids in the cytokine that are located in the receptor-binding interface for replacement residues that include amino acid side-chain conformations that are predicted to fit into the binding interface with the target receptor so as to provide an increase in binding affinity and selectivity/specificity of the cytokine

protein for that target receptor, provided that these are not residues interacting with amino acids that are conserved among receptors bound by the cytokine protein,

so as to provide variants with enhanced stability and increased binding affinity and selectivity/specificity for the target receptor.

- 5 36. A  $\beta$  sheet multimeric cytokine with selectivity for a target receptor whose sequence has been altered by;

a) mutating a residue in a monomer component of the multimeric cytokine protein so as to improve the free energy of the monomer or of the multimeric complex relative to the wild-type unmutated monomer component, wherein said mutated residue is non-conserved  
10 between homologous members of the cytokine family, so as to be more stable than the wild-type, unaltered cytokine protein, and

b) substituting one or more amino acids in the cytokine for replacement residues so as to decrease affinity for one or more different target receptors, provided that these are not residues interacting with amino acids that are conserved among receptors bound by the  
15 cytokine protein

so as to provide variants with enhanced stability and selectivity/specificity for the target receptor.

37. A cytokine according to claim 35 or 36, which is a member of the TNF ligand family.

38. A cytokine according to claim 37, which is TRAIL.

- 20 39. A cytokine according to claim 38, which contains the mutations D269H and T214R.

40. A cytokine according to claim 38, which contains the mutations D269H, E194I and I196S.

41. A computer-implemented method for the stabilisation of a  $\beta$  sheet multimeric cytokine, comprising the step of:

25 mutating a residue in a monomer component of the multimeric cytokine protein so as to improve the free energy of the monomer or of the multimeric complex relative to the wild-type unmutated monomer component;

wherein said mutated residue is non-conserved between homologous members of the cytokine family.

- 30 42. A method according to claim 41, wherein the non-conserved residue that is mutated is at the surface of the monomer component of the multimeric cytokine protein.

43. A method according to claim 41, wherein the non-conserved residue that is mutated is near a position close to the interface between two monomer components of the cytokine protein.
- 5 44. A method according to claim 41, wherein in a trimeric cytokine protein, the non-conserved residue that is mutated is at a position along the central trimeric axis of the multimeric protein.
45. A method according to any one of claims 41-44, wherein more than one non-conserved residue is mutated.
- 10 46. A method according to any one of the preceding claims, wherein non-conserved residues are identified using a computer-implemented alignment algorithm.
47. A method according to claim 64, wherein in an alignment between the candidate for mutation and other members of the same protein family, a conserved residue is one that is shared between at least 50% of the family.
- 15 48. A method according to any one of the preceding claims, wherein a protein design algorithm is used to facilitate the identification of candidate residues for mutation.
49. A method according to claim 48, wherein said method performs an energy calculation involving the following steps:
- 20 a) identification of residues of a monomer that could establish specific interactions with the contiguous monomer;
- b) identification of side chains that contact residues that are candidates for mutation;
- c) at each residue position is placed each amino acid in a repertoire selected from a set of naturally occurring amino acids in a multiple sequence alignment of members of the same protein family, and any side-chain conformations and amino acids that are not compatible with the rest of the structure are eliminated;
- 25 d) all possible pair-wise interactions are explored to eliminate those combinations that are not favourable.
50. A method according to claim 49, wherein said energy calculation is carried computationally, taking into account the properties of the structure, including its atomic contact map, the accessibility of its atoms and residues, the backbone dihedral angles, in addition to the H-bond network and electrostatic network of the protein, the contribution of water molecules making two or more H-bonds with the protein, polar and hydrophobic solvation energies, van der Waals' interactions, van der Waals' clashes, H-bond energies, electrostatics, and backbone and side chain entropies.
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51. A method according to claim 50, wherein the method outputs a sequence and/or PDB coordinates corresponding to the best calculated solution.
52. A method according to claim 51, wherein the sequence and/or PDB co-ordinates including the mutations are energy-minimized and the final predicted energies are compared to the reference, wild-type structure in terms of  $\Delta\Delta G$  (kcal mol<sup>-1</sup>).
53. A method for the alteration of the selectivity of a  $\beta$  sheet multimeric cytokine for a target receptor, the method comprising
- a) identifying amino acids in the cytokine that are located in the receptor-binding interface as candidates for mutation;
- b) discarding residues interacting with amino acids that are conserved among receptors bound by the cytokine protein;
- c) discarding residues interacting with the receptor backbone; and
- d) substituting each of one or more residues in the cytokine protein for replacement residues that include amino acid side-chain conformations that are predicted to fit into the binding interface with the target receptor so as to provide an increase in binding affinity and selectivity/specificity of the cytokine protein for that target receptor.
54. A  $\beta$  sheet multimeric cytokine whose sequence has been altered by a method according to claim 53 so as to alter its affinity for a particular target receptor.
55. A cytokine according to claim 54, which is mutated at one or more of the positions 131, 269, 130, 160, 218, 220, 149, 155, 214, 195, 191 and 267
56. A cytokine according to claim 52 or 53, which is a member of the TNF ligand family.
57. A cytokine according to claim 54, which is TRAIL.
58. A cytokine according to claim 57, which has superior selectivity for the DR5 (TRAIL-R2) or DR4 (TRAIL-R1) over the decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4).
59. A cytokine according to claim 57, which has superior selectivity for the death receptor 5 (TRAIL-R2) over selectivity for the death receptor 4 (TRAIL-R1)
60. A cytokine according to claim 58, which contains one or more of the mutations G131R, D269H, D269K, D269R, R130E, G160K, D218R, G160M, D218Y, D218E, D218K, D218H, I220M, I220H, R149D, R149H, D218F, E155M, T214R, E195R, R191E and D267R.
61. A cytokine according to claim 60, which contains the mutations G160M or D269H.

62. A method for obtaining variants of a  $\beta$  sheet multimeric cytokine with enhanced stability and increased binding affinity and selectivity/specificity for a target receptor comprising the steps of:

5 a) mutating a residue in a monomer component of the multimeric cytokine protein so as to improve the free energy of the monomer or of the multimeric complex relative to the wild-type unmutated monomer component, wherein said mutated residue is non-conserved between homologous members of the cytokine family, and

10 b) identifying amino acids in the cytokine that are located in the receptor-binding interface as candidates for mutation, discarding residues interacting with amino acids that are conserved among receptors bound by the cytokine protein, discarding residues interacting with the receptor backbone; and substituting each of one or more residues in the cytokine protein for replacement residues that include amino acid side-chain conformations that are predicted to fit into the binding interface with the target receptor.